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The Roles of JNK1 and Stat3 in the Response of Head and Neck Cancer Cell Lines to Combined Treatment with All-*trans*-retinoic Acid and 5-Fluorouracil

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We have used a combination of vitamin A (all-*trans*-retinyl palmitate), 5-fluorouracil (5-FU) and radiation to treat human head and neck squamous cell carcinoma (HNSCC). This chemoradiotherapy is called "FAR therapy." In this study we examined the effects of all-*trans*-retinoic acid (ATRA), the active metabolite of vitamin A, and ATRA plus 5-FU on two HNSCC cell lines (YCU-N861 and YCU-H891) to gain insight into the molecular mechanisms of FAR therapy. ATRA at 1 μM (the order of concentration found in HNSCC tumors treated with FAR therapy) inhibited cell proliferation and caused G1 cell cycle arrest in both cell lines. This was associated with a decrease in cyclin D1, an increase in $p27^{Kip1}$ and a reduction in the hyperphosphorylated form of retinoblastoma protein (pRB). With YCU-N861 cells, ATRA also caused a decrease in Bcl-2 and Bcl-X_L and an increase in Bax. Both ATRA and 5-FU activated c-Jun N-terminal kinase (JNK) 1 and the combination of both agents resulted in additive or synergistic activation of JNK1, and also enhanced the induction of apoptosis. The YCU-H891 cells, in which the epidermal growth factor receptor (EGFR)-signal transducer and activator of transcription 3 (Stat3) pathway is constitutively activated, were more resistant to treatments with ATRA, 5-FU and the combination of both agents than YCU-N861 cells. A dominant negative Stat3 construct strongly enhanced the cellular sensitivity of this cell line to 5-FU but not to ATRA. In addition there is evidence that activation of Stat3 is associated with cellular resistance to radiation in HNSCC. Therefore, the addition to FAR therapy of agents that inhibit activation of the Stat3 pathway may enhance the clinical response of patients with HNSCC to FAR therapy.

Key words: Concomitant chemoradiotherapy — Retinoid — Apoptosis — Cyclin D1

Since 1972 the triple combination of 5-FU, vitamin A, and external radiation has been used for the treatment of HNSCC. This concomitant form of chemoradiotherapy is called "FAR therapy." [1–4] The purpose of this combination was to enhance the effect of radiation. At first, 5-FU was combined with γ-radiation as a radiosensitizer. Then, vitamin A was also administered, because vitamin A was thought to improve the permeability of cellular membranes and to increase the uptake of 5-FU into cancer cells.[1, 2, 5, 6] We found that FAR therapy improves the outcome in patients with HNSCC,[3, 4, 7, 8] especially in patients with early-stage laryngeal cancer, high laryngeal preservation and survival rates have been achieved using FAR therapy.[9]

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The abbreviations used are: 5-FU, 5-fluorouracil; ATRA, all-*trans*-retinoic acid; RA, retinoic acid; RAR, retinoic acid receptor; HNSCC, head and neck squamous cell carcinoma; cdk, cyclin-dependent kinase; pRB, retinoblastoma protein; Stat3, signal transducer and activator of transcription 3; JNK, c-Jun N-terminal kinase; EGFR, epidermal growth factor receptor; TGF-α, transforming growth factor-α; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PVDF, polyvinylidene difluoride; HA, hemaggultinin peptide; SIE, sis-inducible element.

Recent studies have demonstrated that RA, the active metabolite of vitamin A, has various anti-cancer effects, including induction of cell differentiation, cell cycle arrest, induction of apoptosis, and chemopreventive activity.[10, 11] Therefore, it is likely that the vitamin A (all-*trans*-retinyl palmitate) administered in the FAR therapy acts through its major metabolite, RA, thus inhibiting cell proliferation, especially when combined with 5-FU and γ-radiation. However, it is not known whether the vitamin A used in FAR therapy leads to an effective concentration of RA within the tumors of the treated patients. To address this problem, we assessed the actual concentrations of ATRA in tumor tissues obtained from patients that had undergone FAR therapy in the study presented here. We then used a similar or lower concentration of ATRA in cell culture studies, employing two HNSCC cell lines, YCU-N861 and YCU-H891. The effects of ATRA alone on proliferation, cell cycle progression, and the levels of cell cycle and apoptosis related proteins were also evaluated.

In a previous study we found that the triple combination of ATRA, 5-FU and radiation synergistically induces apoptosis following G1 arrest in HNSCC cells.[12] However, the precise mechanism by which ATRA, 5-FU and radia-

tion synergize to induce cell death is not known. Persistent activation of the serine/threonine kinase JNK1 is frequently associated with apoptosis induced by several agents,[13] including γ-radiation,[14, 15] 5-FU,[16] and synthetic retinoids.[17, 18] Therefore, in the present study we also examined the combination effects of ATRA and 5-FU on JNK1 activation.

Patients with HNSCC differ in their sensitivity to FAR therapy and some are highly resistant, regardless of their clinical stage. A number of studies have indicated that overexpression of the EGFR is an adverse prognostic factor in this disease.[19] Recent studies have demonstrated that Stat3 is a major target of the EGFR and that activation of this transcription factor plays an important role in the progression of HNSCC and in protecting cancer cells from apoptosis.[20, 21] Thus, constitutive activation of Stat3 may play a role in cellular resistance to FAR therapy. Therefore, in the present study we examined the effects of a dominant negative Stat3 construct on cellular sensitivity to ATRA and 5-FU, employing YCU-H891 cells, in which we found that the EGFR-Stat3 pathway is constitutively activated.

MATERIALS AND METHODS

Assays of ATRA in tumor tissue samples Six samples were obtained from patients with carcinoma of oral pharynx who had undergone 10 cycles of FAR therapy. The total amount of vitamin A ("Chocora-A"; all-*trans*-palmitate ester of retinol, Eisai Co., Tokyo) administered at that point was 500 000 IU. The tissue samples were immediately frozen in liquid nitrogen and stored at −80°C. Analyses for ATRA concentration in tumor samples were done by Pharmacokinetics Research Laboratory, Taiho Pharmaceutical Co., Ltd. (Tokushima).

Chemicals and drugs ATRA (Sigma, Tokyo) was dissolved in ethanol and was stored in the dark at −80°C. In all experiments the final concentration of ethanol added to the cell culture medium was less than 0.1%. 5-FU was provided by Kyowa Co. (Tokyo).

Cell lines and cell culture The human head and neck cancer cell lines, YCU-N861 (nasopharynx) and YCU-H891 (hypopharynx) were generously provided by Dr. M. Tsukuda (Yokohama City University). These cells were maintained in a 5% CO_2 atmosphere at 37°C in RPMI-1640 medium with 10% fetal bovine serum, plus 1% penicillin and 1% kanamycin (Life Technology, Tokyo).

Proliferation assays Ten thousand cells of each cell line were seeded into 35-mm dishes and treated with the indicated concentrations of ATRA for 96 h. As a negative control, 0.1% ethanol alone was used. The numbers of cells were then determined in triplicate plates using a Coulter Counter (Beckman Coulter, Tokyo) and the growth rates were determined.

Cell viability assays Cell viability was determined using a PreMix WST-1 Assay System (TaKaRa, Tokyo) according to the manufacturer's instructions. Briefly 3×10^3 cells were seeded in a 96-well plate. Twenty-four hours later the indicated concentration of drugs were added. After treatment for 72 h, cells were incubated for 2 h in the medium containing 10% WST-1 reagent and then the absorbance was measured using a microplate reader at a wavelength of 450 nm. The cell viability was calculated by dividing the absorbance of treated cells by that of the respective control cells (taken as 1.0). Each point represents the average of triplicate wells.

Cell cycle assays Exponentially growing cells were treated with the indicated concentrations of drugs for 72 h and harvested by trypsinization. The cells were washed twice with PBS, fixed in 70% ethanol for 30 min at room temperature, washed with PBS, treated with 0.1 μg/ml RNAse, and stained with 25 μg/ml propidium iodide. Flow cytometric analysis was carried out using an "EPICS" XL (Beckman Coulter) or FACScalibur (Becton Dickinson, San Diego, CA) instrument.

Protein extraction and western blot analysis Control or treated cells were harvested by trypsinization. Total cellular protein was extracted and quantified using the M-Per Mammalian Protein Extraction Reagent and "Coomassie" Protein Assay Reagent Kit (Pierce, Rockford, IL). The quantification was repeated twice. For immunoblotting, 20−30 μg per lane of protein were loaded and electrophoresed on a 10−12.5% SDS-polyacrylamide gel, and then transferred onto PVDF membranes. The following primary antibodies were used: RARα (D-20), RARβ (C-20), RARγ (C-19), $p21^{Cip1}$ (H-164), cyclin D1 (M-20), pRB (SC-50), Bcl-2 (N-19), Bcl-X_L (H-62) and Bax (N-20), from Santa Cruz, Santa Cruz, CA; p16 (DCS-50) and $p27^{Kip1}$ (DCS-72) from Oncogene, Cambridge, MA; cdk4 (06-139) from Upstate Biotechnology, Lake Placid, NY; and actin (20-33) from Sigma, St. Louis, MO. Specific protein bands were detected using the ECL-enhanced chemiluminescence system (Amersham International, Buckinghamshire, UK). NIH image (version 1.6) was used for densitometric quantitation.

Assays for the activation of EGFR, Stat3 and JNK Cells were cultured under the indicated conditions and then proteins were extracted using the above-mentioned method. The levels of phospho and total cellular EGFR, Stat3 and JNK1 proteins were determined by western blot analysis with the respective phospho-specific and non-phospho-specific antibodies. JNK1 activity was measured by densitometric analysis of the phosphorylated form of the JNK1 proteins. The following antibodies were used; EGFR (clone-13) and phospho-EGFR (clone-74) from Transduction Laboratory, Lexington, KY; Stat3 (F-2) and phospho-Stat3 (B-7) and total JNK (sc-571) from Santa Cruz; and phospho-JNK (V7931) from Promega, Madison, WI.

Luciferase reporter assays The cyclin D1 luciferase reporter plasmid -1745CD1LUC was constructed and provided by Dr. R. Pestell (Albert Einstein Cancer Center).[22] The c-*fos* promoter luciferase reporter plasmid p-FOS-wt-luc, and the method for transfection luciferase reporter assays, were described previously.[23] Triplicate samples of 1×10^5 cells in 35-mm plates were transfected with lipofectin (Life Technology, Grand Island, NY) in the serum minus medium. One microgram of the reporter plasmid and 10 ng of pCMV-β-gal plasmid DNA were cotransfected. After 16 h, 1 μM ATRA or the ethanol solvent was added. The cells were then incubated for 24 h and luciferase activity was determined with the luciferase assay system (Promega). The β-gal activities were determined with a β-galactosidase enzyme assay system (Promega). Luciferase activities were then normalized with respect to β-gal activities.

Stable transfection with dominant-negative Stat3 The dominant-negative HA-tagged-Stat3D construct, in which the glutamic acids 434 and 435 in the DNA binding site were replaced with alanines, was constructed and provided by Dr. T. Hirano (Osaka University).[24] The Stat3D plasmid contains an internal neomycin resistance marker and has been used in previous studies.[21, 25] Cells were transfected as described above and several stably transfected clones were selected in medium containing 400 μg/ml of G418.

Immunoprecipitation and immunoblotting Immunoprecipitation was performed essentially as previously described.[21] Briefly, 100 μg of cell lysates were immunoprecipitated with the anti-Stat3 antibody (F-2, Santa Cruz), coupled to protein A/G plus agarose (L119, Santa Cruz), and then immunoblotted with an anti-HA antibody (Y-11, Santa Cruz) or with an anti-Stat3 antibody.

Statistical analyses Statistical analyses were performed using one-way ANOVA methods. Differences were considered to be statistically significant when the *P* value was less than 0.05.

RESULTS

Concentrations of ATRA in tumor tissue The average concentration of ATRA±SD in 6 tumor samples obtained from patients who had undergone 10 cycles of FAR therapy was 0.64±0.013 μmol/kg (range, 0.43 to 0.8 μmol/kg). Therefore, we decided that the maximum concentration of ATRA for use in our cell culture studies would be 1 μM (10^{-6} *M*).

Expression of RARs and phosphorylated forms of EGFR and Stat3 We used western blot analyses to characterize the levels of specific RARs and phosphorylated forms of EGFR and Stat3 in the two HNSCC cell lines. We found that the nasopharyngeal carcinoma cell line YCU-N861 expressed both RARα and RARβ, but not RARγ. In contrast the hypopharyngeal carcinoma cell line YCU-H891 cell strongly expressed RARγ, but only weakly expressed RARα and RARβ (Fig. 1A). The status of the EGFR-Stat3 pathway was also determined by western blot analyses (Fig. 1B). Cells were cultured in serum minus medium for 48 h to reduce exposure to external growth stimulation, and then half the cultures were stimulated with TGF-α for 6 h and proteins were extracted. In YCU-N861 cells, both the total cellular and the phosphorylated forms of the EGFR and Stat3 proteins were observed only after stimulation with TGF-α (Fig. 1B). In contrast, YCU-H891 cells displayed relatively high levels of the total cellular EGFR and Stat3 proteins in the presence or absence of TGF-α. In addition, YCU-H891 cells expressed relatively high level of phospho-EGFR and Stat3 proteins even after 48 h of serum starvation, and stimulation with TGF-α did not increase the levels of these two phospho-proteins (Fig. 1B). These results indicate that in YCU-H891 cells the EGFR-Stat3 pathway is constitutively activated, while in YCU-N861 cells activation of this pathway is dependent on exogeneous stimulation by TGF-α.

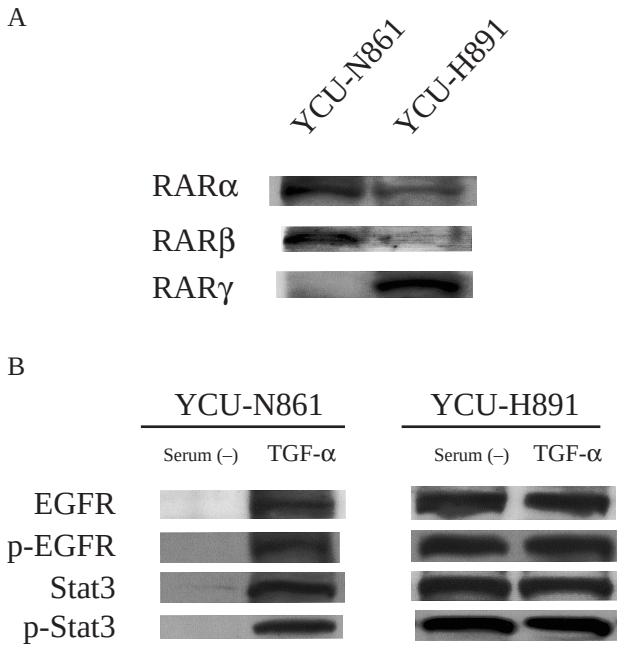

Fig. 1. Western blot analyses for retinoic acid receptors α, β and γ (A), and total cellular and phosphorylated forms of the EGFR and Stat3 proteins (B) in the YCU-N861 and YCU-H891 cell lines. In B, cells were cultured in the serum minus medium for 48 h and half of the cultures were stimulated with 50 ng/ml of TGF-α for 6 h, then proteins were prepared. p-EGFR and p-Stat3 indicates phosphorylated forms of the EGFR and Stat3 proteins, respectively.

ATRA inhibits growth in both of the HNSCC cell lines To assess the effects of ATRA alone on the proliferation of the two HNSCC cell lines, cells were treated with 1 μM or 1 nM ATRA for 96 h (Fig. 2). ATRA significantly ($P<0.0001$) inhibited proliferation of both cell lines in a dose-dependent manner (Fig. 2). However, the growth-suppressive effects were more striking in YCU-N861 cells than in YCU-H891 cells (Fig. 2).

ATRA induces cell cycle arrest in G1 Cell cycle assays using DNA flow cytometry were carried out to gain insight into the mechanism of growth inhibition by ATRA (Fig. 3A). Treatment with 1 μM ATRA for 72 h induced a cell cycle arrest in G1, in both the YCU-N861 and YCU-H891 cell lines. The percent of cells in the G1 phase increased from 63.0% to 85.7% in the YCU-N861 cells and from 66.5% to 81.1% in the YCU-H891 cells (Fig. 3A). No significant increase in the sub G1 fraction was seen in either cell line after treatment with ATRA in this assay.

ATRA alters the levels of specific cell cycle control proteins In view of the effects of ATRA on cell cycle progression, we examined the effects of a 72 h treatment with 1 μM ATRA on the expression of specific cell cycle proteins, using western blot analyses. The $p16^{INK4A}$ and $p21^{Cip1}$ proteins were not detected in either of these two cell lines before or after treatment with ATRA (data not shown). Treatment with ATRA caused a marked decrease in the level of the cyclin D1 protein, in both cell lines. This effect was strongest in the YCU-N861 cell line (Fig. 3B). Induction by ATRA (about 2.0 fold) of the $p27^{Kip1}$ protein was seen in the YCU-N861 cell, but not in the YCU-H891 cell line (Fig. 3B). The levels of cyclin E and cdk4 remained unchanged in both cell lines after treatment with ATRA (data not shown). In both cell lines we observed a slight decrease in the phosphorylated form of the pRB protein (Fig. 3B). Presumably the increase of cells in G1 noted in Fig. 3A reflects the decrease in the level of the cyclin D1 protein and the increase in the level of the $p27^{Kip1}$ protein.

ATRA does not directly inhibit cyclin D1 promoter activity Since ATRA caused a decrease in the cellular level of the cyclin D1 protein (Fig. 3B), we examined the effects of ATRA on cyclin D1 promoter activity, using a cyclin D1 promoter-luciferase reporter in transient transfection assays. Treatment with 0.1 μM or 1 μM ATRA did not influence this luciferase activity in either of the cell lines (data not shown), suggesting that the ATRA-mediated cyclin D1 downregulation shown in Fig. 3B was not due to inhibition of *de novo* transcription of this gene.

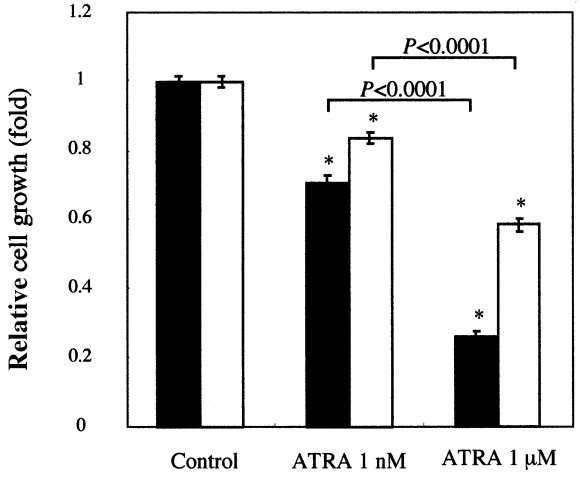

Fig. 2. Relative cell growth of YCU-N861 and YCU-H891 cells. Cells were treated with 1 nM or 1 μM ATRA, or ethanol vehicle alone (control) for 96 h. Similar results were obtained in a repeat experiment. Brackets indicate SD. *, $P<0.0001$ as compared with control. ■ YCU-N861, □ YCU-H891.

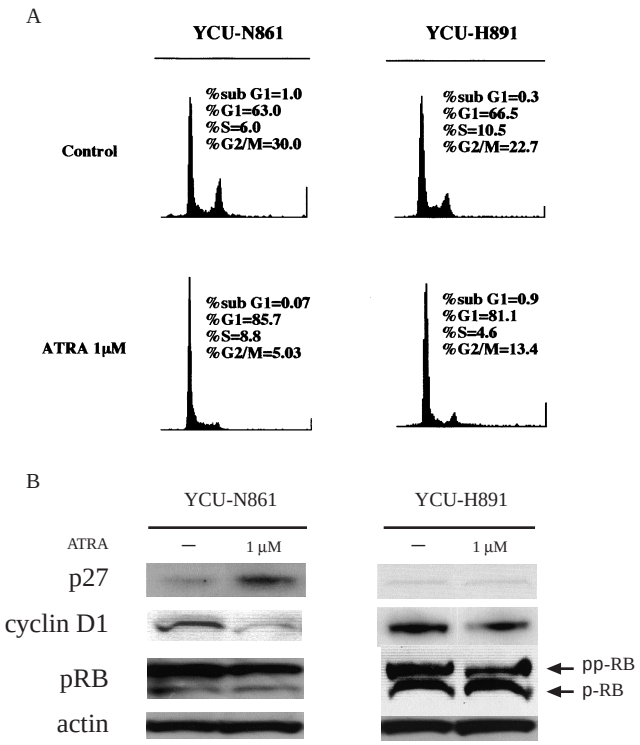

Fig. 3. Flow cytometric analysis (A), and western blot analysis for the $p27^{Kip1}$, cyclin D1 and pRB proteins (B), in YCU-N861 and YCU-H891 cells after treatment with ATRA. A) exponentially growing cells were grown in the presence of 1 μM ATRA or ethanol alone (control) for 72 h and then analyzed by DNA flow cytometry. B) cells were treated with 1 μM ATRA or ethanol vehicle alone (−) for 72 h. Extracts were then prepared and examined by western blot analyses with the respective antibodies. An antibody for actin was used as a loading control. pp-RB, hyperphosphorylated form of p-RB protein.

The combination of ATRA and 5-FU showed enhanced inhibitory effects on cell viability in both YCU-N861 and YCU-H891 cells To assess the effect of ATRA alone, 5-FU alone and the combination of both agents, cell viability assays were conducted (Fig. 4A). Cells were treated with 1 μM ATRA alone, 1 μg/ml 5-FU alone or the combination of both agents, for 72 h, and cell viability was determined. Treatment with 1 μM ATRA alone for 72 h did not inhibit cell viability with YCU-H891 cells, but it did inhibit cell viability by 20% ($P<0.01$) with YCU-N861 cells (Fig. 4A). Treatment with 5-FU alone caused an 80% ($P<0.0001$) decrease in cell viability with YCU-N861 cells and a 20% (not significant) decrease with YCU-H891 cells (Fig. 4A). The combination of 1 μM ATRA plus 1 μg/ml 5-FU showed enhanced cytotoxic effects, inhibiting cell viability 90% ($P<0.0001$) with YCU-N861 cells and 50% ($P<0.01$) with YCU-H891 cells (Fig. 4A). Thus, the YCU-H891 cells were more resistant to treatment with ATRA alone, 5-FU alone and the combination of both agents than the YCU-N861 cells (Fig. 4A).

ATRA alters the levels of Bcl-family proteins in YCU-N891 cells, but not in YCU-H891 cells With the YCU-N861 cell line, treatment with 0.01, 0.1 or 1 μM ATRA for 72 h caused a decrease in the levels of the antiapoptotic proteins Bcl-2 and Bcl-X_L and an increase in the level of the proapoptotic protein Bax, in a dose-dependent manner (Fig. 5A). Densitometric analysis results for these proteins are summarized in Fig. 5B. The left panel shows the relative level of each protein. The right panel shows the Bcl-2/Bax or Bcl-X_L/Bax ratios. In the YCU-N861 cells, treatment with ATRA caused decreases in the ratios of the Bcl-2/Bax and Bcl-X_L/Bax proteins in a dose-dependent manner (Fig. 5B). In the YCU-H891 cell line the levels of these three proteins remained unchanged following treatment with ATRA (Fig. 5A). The decline in the ratios of the Bcl-2/Bax and Bcl-X_L/Bax proteins in YCU-N861 cells might explain, at least in part, the greater inhibitory effects of ATRA on growth and cell viability in this cell line (Figs. 2 and 4A).

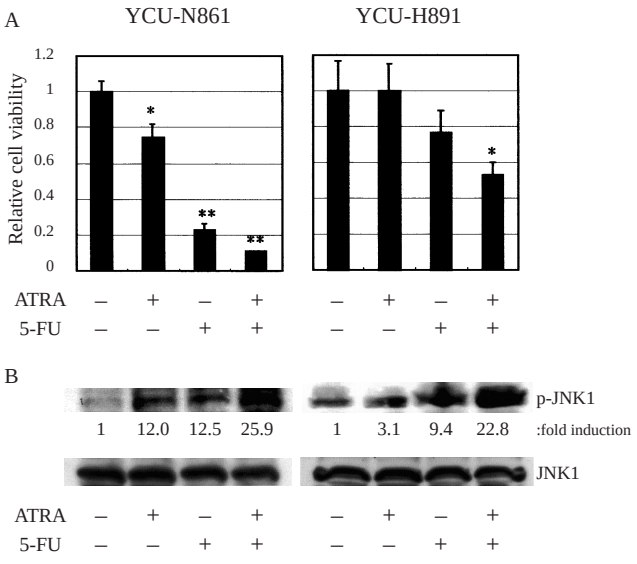

Fig. 4. Cell viability assays (A), and western blot analysis for phospho-specific JNK1 and total cellular JNK1 (B). A) cells were treated with 1 μM ATRA alone, 1 μg/ml 5-FU alone or the combination of the both agents, for 72 h, and cell viability was measured by using cell proliferation assays. B) cells were treated with 1 μM ATRA alone, 10 μg/ml 5-FU alone or the combination of the both agents, for 6 h, and then proteins were extracted and examined by western blot analyses with the respective antibodies. Densitometric analysis for the phospho-JNK1 proteins was performed and fold induction was calculated. Brackets indicate SD. *, $P<0.01$ and **, $P<0.0001$ as compared with control.

ATRA enhances the apoptosis caused by 5-FU In view of the enhanced inhibitory effects on cell viability by the combination of ATRA and 5-FU (Fig. 4A), we examined the effects of this combination on cell cycle progression and apoptosis, using DNA flow cytometry (Fig. 6). In YCU-N861 cells, treatment with 50 μg/ml of 5-FU alone for 72 h induced a marked G1 cell cycle arrest, but no significant increase in the sub G1 population (Fig. 6). In contrast, when cells were treated with both ATRA (0.1 or 1 μM) and 50 μg/ml 5-FU, there was a prominent sub G1 cell population, which increased with the higher dose of ATRA (Fig. 6). The increases in sub G1 in the 5-FU plus ATRA treated cells, when compared to cells treated with 5-FU alone, were highly significant ($P<0.0001$). In YCU-H891 cells, similar results were obtained but the increase in the sub G1 population was not statistically significant (data not shown).

ATRA plus 5-FU causes synergistic activation of JNK1 Since there is evidence that activation of JNK1 is associated with both 5-FU and retinoid induced-apoptosis in other cell systems,[16–18] we analyzed the effects of treating our HNSCC cell lines with 1 μM ATRA alone, 1 μg/ml 5-FU alone, and the combination of the both agents on JNK1 activation. The cells were treated for 6 h and cell extracts were examined by western blot analysis, using both phosphospecific and non-phosphospecific JNK antibodies (Fig. 4B). Densitometric analyses for the phospho-JNK1 proteins were then performed. Treatment with ATRA alone caused 12.0-fold increase in the level of the phospho-JNK1 protein with YCU-N861 cells, and 3.1-fold increase with the YCU-H891 cells, when compared to the control untreated cells (taken as 1.0) (Fig. 4B). Treatment with 5-FU alone also increased the level of the phospho-JNK1 protein in both cell lines, resulting in a 12.5-fold increase with the YCU-N861 cells and a 9.4-fold increase with the YCU-H891 cells (Fig. 4B). The combination of ATRA plus 5-FU caused additive (YCU-N861) or syner-

gistic (YCU-H891) activation of JNK1 (Fig. 4B). Thus, YCU-N861 cells showed a 25.9-fold increase in phospho-JNK1 and YCU-H891 cells a 22.8-fold increase (Fig. 4B). There were no significant changes in the levels of the total cellular JNK1 protein (Fig. 4B). These results suggest that the combination of ATRA plus 5-FU causes enhanced cytotoxic effects because of the additive or synergistic effects in JNK1 activation.

A dominant-negative Stat3 construct enhances cellular sensitivity of YCU-H891 cells to 5-FU, but not to ATRA As demonstrated in Fig. 1B, the EGFR-Stat3 pathway is constitutively activated in YCU-H891 cells, but not

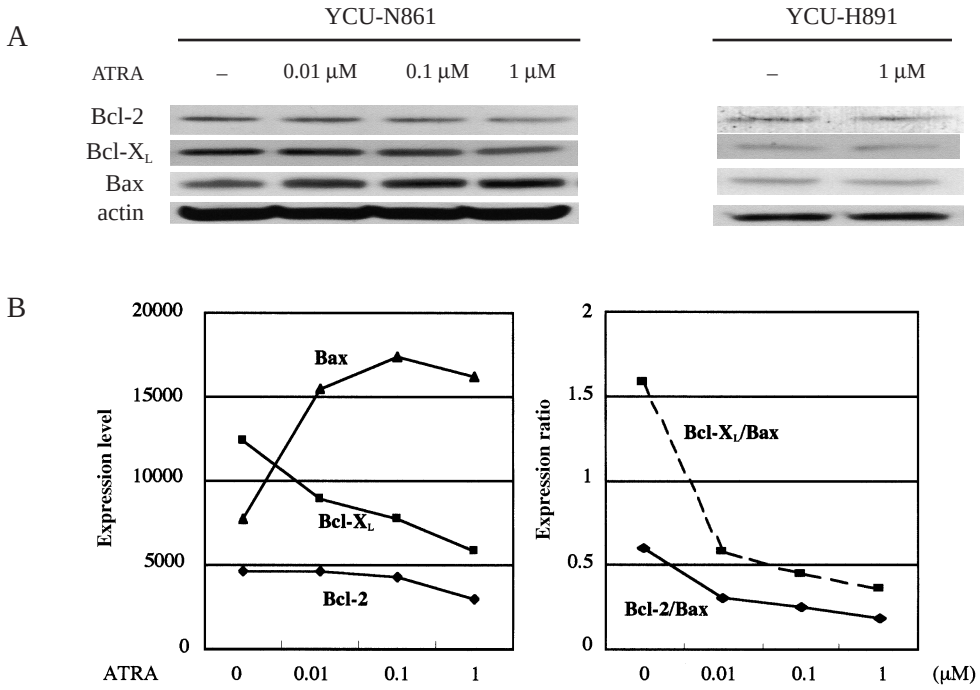

Fig. 5. Western blot analysis for the Bcl-2, Bcl-X_L and Bax proteins in YCU-N861 and YCU-H891 cells (A), and densitometric analysis of the abundance of the Bcl-2, Bcl-X_L and Bax proteins in YCU-N861 cells (B). A) cells were treated for 72 h with the indicated concentrations of ATRA or with 0.1% ethanol (−). Extracts were then prepared and examined by western blot analyses, with the respective antibodies. An antibody for actin was used as a loading control. B) the levels of specific bands with YCU-N861 cells in panel A were quantificated. The left panel shows the relative level of each protein. The right panel shows the Bcl-2/Bax or Bcl-X_L/Bax ratios.

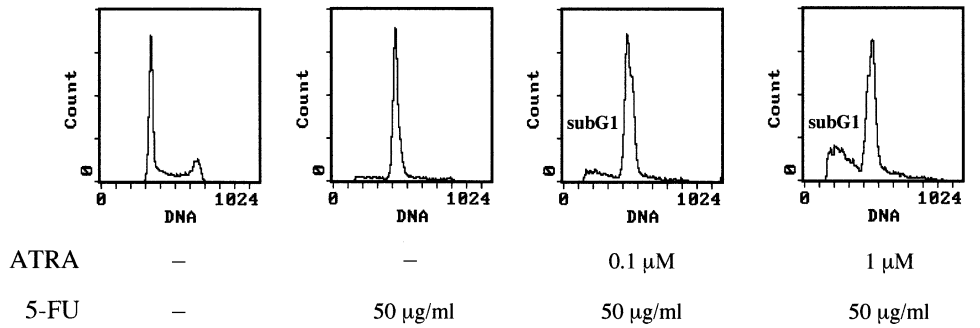

Fig. 6. Assays for effects on the cell cycle and on apoptosis of treatment with ATRA plus 5-FU. Exponentially dividing cultures of YCU-N861 cells were treated for 72 h with the indicated concentrations of drugs. The cells were then analyzed by DNA flow cytometry. The extent of apoptosis was determined by the size of sub G1 fraction.

YCU-N861 cells. The former cell line is also more resistant to treatment with ATRA alone (Figs. 2 and 4A), 5-FU alone (Fig. 4A), and the combination of ATRA and 5-FU (Figs. 4A and 6) than YCU-N861 cells. These results are consistent with findings of previous investigators that Stat3 activation protects cancer cells from apoptosis.[21, 26] Therefore, it was of interest to analyze the effects of a dominant-negative Stat3 construct on cellular responses of YCU-H891 cells to ATRA alone, 5-FU alone and the combination of both agents. We established two clones that stably express relatively high levels of a dominant-negative HA-tagged-Stat3 protein (see "Materials and Methods") and named them Stat3DN66 and Stat3DN99 (Fig. 7A). As expected, the HA-tagged-Stat3D protein was detected in these two clones, but not in parental YCU-H891 cells or in empty vector-transfected cells (Fig. 7A). Since the c-*fos* promoter contains the SIE sequence[23] which has a high affinity for the Stat3 protein,[27] we examined c-*fos* promoter activity using a c-*fos* promoter-luciferase-reporter in transient transfection assays. The c-*fos* promoter activity was strongly inhibited in the Stat3DN66 and Stat3DN99 cells, compared to the parental cells and the vector control clone, providing evidence that the HA-tagged-Stat3D protein functions as dominant negative in this system (Fig. 7B). We treated the parental YCU-H891, vector control, Stat3DN66 and Stat3DN99 cells with 1 μM ATRA alone, 1 μg/ml 5-FU alone, and the combination of both agents for 72 h, and then cell viability was determined (Fig. 7C). In the parental and vector control cells, ATRA alone did not inhibit cell viability, but 5-FU alone or the combination of ATRA plus 5-FU inhibited cell viability 20% (not significant) and 40% ($P<0.01$), respectively (Fig. 7C). Thus, ATRA enhanced the inhibitory effects of 5-FU by 20% ($P=0.06$). These results are consistent with those obtained in Fig. 4A. With both of the Stat3 dominant-negative clones, ATRA alone also did not inhibit cell viability, but treatment with 5-FU alone strongly inhibited cell viability, by 70% ($P<0.0001$) in both cell lines. It is of interest that the addition of ATRA to 5-FU did not further enhance the inhibitory effects of 5-FU in these two clones (Fig. 7C). However, as a result the cytotoxic effects of 5-FU plus ATRA were greater in the dominant-negative Stat3 clones than in the parental or control cells (Fig. 7C). These results suggest that abrogation of Stat3 activity enhances cellular sensitivity of YCU-H891 cells to 5-FU but not to ATRA.

DISCUSSION

One cycle of FAR therapy consists of intravenous injections of 5-FU (250 mg/day), intramuscular vitamin A (50 000 IU/day), and radiation (1.5–2.0 Gy/day).[3] This combination is administered once a day, 5 times per week.[3] Patients receive 15 to 40 cycles of FAR therapy.[3, 4, 7–9, 28] In the present study, we found that even at the early phase of FAR therapy (10 cycles), concentrations of ATRA within tumors reach a relatively high level, about 1 μM. This concentration of ATRA caused inhibi-

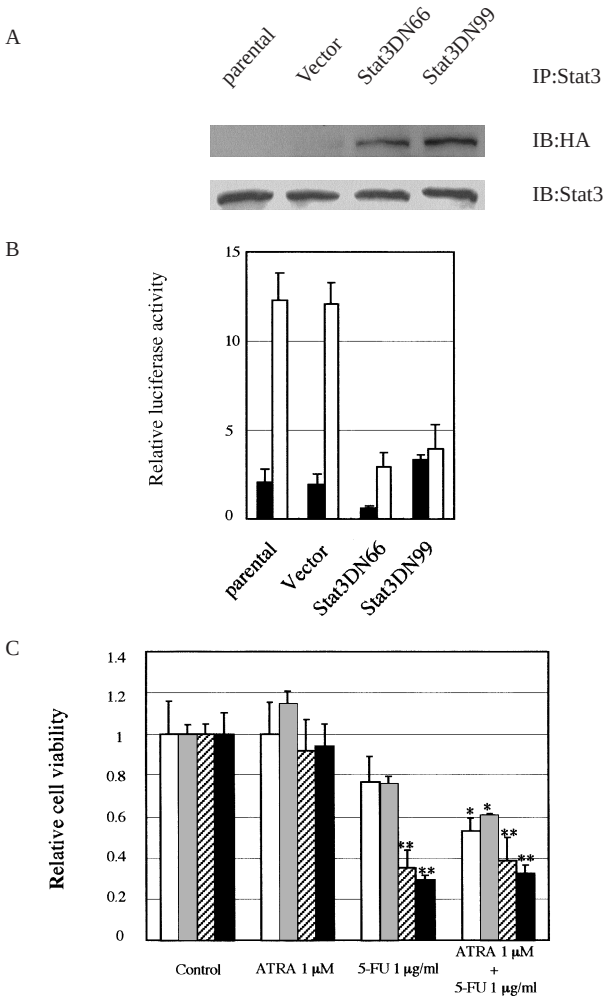

Fig. 7. Immunoprecipitation and immunoblotting assays (A), c-*fos* promoter luciferase reporter assays (B), and cell viability assays (C), for parental YCU-H891 cells, an empty vector transfected clone, and two dominant-negative Stat3 transfected clones. A) total cellular lysates were immunoprecipitated with an anti-Stat3 antibody and then immunoblotted with an anti-HA antibody or anti-Stat3 antibody. B) cells were transfected in "opti-MEM" I medium with the c-*fos*-promoter-luciferese reporter plasmid for 16 h. Then the medium was changed to serum minus RPMI-1640 medium and the cells were incubated for an additional 24 h in the absence (■) or presence (□) of TGF-α. C) cells were treated with 1 μM ATRA alone, 1 μg/ml 5-FU alone or the combination of both agents, for 72 h, and then assayed for cell viability. Brackets indicate SD. □ YCU-H891, □ vector, ▨ dominant-negative 66 and ■ dominant-negative 99. *, $P<0.001$ and **, $P<0.0001$ as compared with control.

tion of growth of both the YCU-N861 and the YCU-H891 HNSCC cell lines (Fig. 2), and this was associated with arrest of the cells in the G1 phase of the cell cycle (Fig. 3). Western blot analyses demonstrated that treatment with ATRA caused a decrease in the cellular level of cyclin D1 in both cell lines, an increase in the level of the cell cycle inhibitor $p27^{Kip1}$ in the YCU-N861 cells and a decrease in the phosphorylated form of pRB in both cell lines (Fig. 3B). These changes presumably account for the G1 cell cycle arrest and growth inhibition caused by ATRA. However, it is curious that in the YCU-H861 cells, the effects on cyclin D1 and pRB were less striking and there was no induction of $p27^{Kip1}$ (Fig. 3B). Therefore, in this cell line ATRA may act through other mechanisms to arrest the cell cycle in G1. Previous investigators also reported that RA can cause arrest of the cell cycle in G1, a decrease in the level of the cyclin D1 protein and an increase in the level of the $p27^{Kip1}$ proteins in various types of cancer cells.[29–37] Our cyclin D1 promoter-luciferase reporter assays suggest that the decrease in cyclin D1 protein in ATRA-treated HNSCC cells is due to post-transcriptional effects. This is consistent with studies which indicate that in bronchial epithelial cells ATRA causes a decrease in the cyclin D1 protein via a ubiquitin-mediated pathway.[31, 35] In addition, the cellular levels of $p27^{Kip1}$ are usually regulated at the level of proteolysis, via a ubiquitin-mediated pathway.[38] Thus, the increase in $p27^{Kip1}$ we found in ATRA-treated YCU-N891 cells (Fig. 3B) may be due to a suppression of this protease pathway, although this remains to be determined. It is of interest that a decreased level of $p27^{Kip1}$ and an increased level of cyclin D1 are often associated with a poor prognosis in various types of human cancer,[38, 39] including HNSCC.[28, 40, 41] Therefore the increase of $p27^{Kip1}$ and decrease in cyclin D1 in ATRA-treated HNSCC cells may contribute to the therapeutic effects of ATRA in FAR therapy.

The inhibitory effects of ATRA on cell growth and viability were greater in the YCU-N861 cells than in the YCU-H891 cells (Figs. 2 and 4A). The decreased level of expression of RARβ in the YCU-H891 cells (Fig. 1A) may, at least in part, explain their relative resistance to ATRA. Repression of RARβ expression has been seen in oral premalignant lesions and in several malignancies that are resistant to retinoids.[42, 43] Furthermore, overexpression of RARβ in a HNSCC cell line which otherwise expresses a low level of RARβ, enhances RA-induced apoptosis, and this is associated with an increase in the level of the Bax protein and a decrease in the level of the Bcl-X_L protein.[44] These results correlate with our finding that in YCU-N861 cells, which express relatively high levels of RARβ, ATRA caused decreases in the levels of the Bcl-2 and Bcl-X_L proteins and an increase in the level of the Bax protein (Fig. 5), and that these cells are more sensitive to inhibition of cell growth and viability by ATRA (Figs. 2 and 4A). However, in HNSCC the roles of RARs in response to RA are still controversial, since several reports demonstrated that RARγ rather than RARβ is required for *in vitro* growth inhibition of HNSCC by RA.[45–47] Therefore, in future studies it will be important to more precisely evaluate the role of each RAR in FRA therapy.

In the present study we also found that ATRA enhanced the cytotoxic effects of 5-FU in both of the HNSCC cell lines (Figs. 4A and 6). The synergistic interactions between ATRA and 5-FU seen in the present study are consistent with several previous studies indicating that ATRA can potentiate the effects of various cytotoxic agents, including *cis*-platinum, on HNSCC and ovarian adenocarcinoma cells,[48] 5-FU on epidermoid cells,[49] adriamycin and paclitaxel on breast cancer cells,[50] and radiation on skin fibroblasts and melanoma cells.[51] Although the precise mechanisms by which ATRA enhances the cytotoxic effects of these agents remains undefined, recent studies provide evidence that *cis*-platinum,[52] 5-FU,[16] paclitaxel,[53] radiation[14, 15, 54] and retinoic acids[17, 18, 55] activate JNK1 and that JNK1 activation can induce apoptosis. In the present study we found that in both of our HNSCC cell lines both ATRA alone and 5-FU alone can activate JNK1, and that the combination of these agents causes synergistic activation of JNK1 (Fig. 4B). These findings may explain, at least in part, the enhanced cytotoxity we observed between ATRA and 5-FU in the present study (Figs. 4A and 6), and the synergistic effects of ATRA, 5-FU and γ-radiation on induction of apoptosis seen in our previous study.[12] Thus, additive or synergistic effects between these agents with respect to JNK1 activation may be relevant to the clinical efficacy of FAR therapy in the treatment of HNSCC.

In the present study the YCU-H891 cells were relatively resistant to the growth inhibitory and cytotoxic effect of ATRA alone and 5-FU alone (Figs. 2 and 4A), and showed a weaker response to the combination of 5-FU and ATRA (Fig. 4A), than the YCU-N861 cells. We hypothesized that the constitutive activation of Stat3 in YCU-H891 cells (Fig. 1B) may be related to this resistance. Indeed, we found that abrogation of Stat3 activity in this cell line with a dominant-negative Stat3 construct markedly enhanced cellular sensitivity to 5-FU, although the response to ATRA alone remained unchanged and ATRA did not enhance the cytotoxic effects of 5-FU in these cells (Fig. 7C). These results suggest that the mechanism of relative resistance to 5-FU in the YCU-H891 cells is, at least in part, caused by activation of Stat3 and that the enhanced cytotoxic effects of 5-FU by ATRA were abrogated by dominant-negative Stat3 construct. As previously reported by several investigators,[56–58] ATRA might enhance the cytotoxic effects of 5-FU through inhibition of EGFR signaling. However, these inhibitory effects are relatively weak. Therefore, when activation of Stat3 is strongly

inhibited by the dominant-negative Stat3 construct, ATRA might not significantly enhance the cytotoxic effects of 5-FU.

A recent study provides evidence that activation of Stat3 by the EGFR protects HNSCC cells from apoptosis induced by radiation, indicating that the activation of Stat3 is associated with cellular resistance to radiation.[59] This result, taken together with our finding that inhibition of Stat3 can enhance cellular sensitivity to 5-FU, suggests that agents that inhibit activation of the Stat3 pathway may be useful in increasing the response to therapy of cases of HNSCC that are currently resistant to FAR therapy.

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